12-6-2010

Standard Protocols for the 3 MST Markers Evaluated

Microbial Source Tracking

Microbial Source Tracking Standard Operating Procedures
Binary PCR Protocol for Human-Associated *Bacteroides, Methanobrevibacter smithii* and Human Polyomaviruses

Scope of Application
This protocol applies to the extraction of microbial DNA from membrane filters followed by amplification of this DNA by binary (conventional PCR) for microbial source tracking analysis.

Summary
This protocol outlines the procedure developed for the EPA Gulf of Mexico grant to Harwood, Lepo and Wang to process environmental water samples via DNA extraction from membrane filters. DNA is then used in PCR-based microbial source tracking assays for human-associated *Bacteroides* (HBac), *M. smithii* (Msmithii) and human polyomavirus BK and JC (HPyVs) targets.

Sample filtration

Note: One DNA extraction is performed to yield DNA template for all three assays: human *Bacteroides*, *M. smithii* and HPyV PCR protocols.

Field/method blank. Process 500 ml of sterile buffered water through all procedures to test for contamination from environmental and/or laboratory sources. A sealed container with sterile water that is transported into the field where environmental samples are collected and then back to the lab is called a **field** blank. It is processed as a sample (filtration, DNA extraction, PCR). If the samples being processed originate from the lab (e.g. buffered water spiked with sewage), then a **method** blank (500 ml of sterile buffered water) should be processed.

- Pour 500 ml of the collected environmental water sample or control sample into a sterile one-liter beaker covered with aluminum foil and containing a sterile magnetic stir bar.

- Adjust water sample pH to 3.5 using 20% HCl (concentrated HCl diluted 1:5), while stirring.
  (Lowering the pH gives the viral capsids a net positive charge. As the water sample is passed through a 0.45 μm nitrocellulose filter, which has a net negative charge, the viruses bind to the filters via electrostatic interactions.)

  - Preferably use pH paper! A narrow range pH paper (0.5 to 5.5, Whatman#2600-101A) can be purchased.
    Or, if pH paper is not available….
  - The pH probe used should be disinfected, and should be re-disinfected between samples:

- Immerse probe in 10% bleach solution (~30 sec)
- Rinse probe with sterile tap water
- Immerse probe in 0.5% sodium thiosulfate solution
- Rinse probe with sterile tap water

- Filter sample through a 0.45 µm pore nitrocellulose filter.

  - If the sample begins to clog the filter, limit the time of filtering to 15 min and record how much volume was filtered. Try to get at least 300ml processed if possible (always record in your results). The turbidity of some water samples may preclude sampling of the full volume.

  - OR, if the filter is clogging, use another 0.45 µm filter to finish the sample, then process the filters as two separate DNA extractions in MoBio PowerSoil Kit, but combine the final eluted DNA. Do not do this step if your sample will take more than **2 filters**. To combine the DNA, filter both through the same spin column in step #11 of the extraction protocol below.

- Using flamed (sterile) forceps, that were previously cleaned with a 10% bleach solution or DNA Away (commercial product designed to remove nucleic acids), fold the 0.45 µm filter (while still on funnel, keep folding it in half) and place into a bead tube from the Mo Bio PowerSoil DNA extraction kit. It takes two pairs of forceps; we have a short video clip of the filter folding we can email.

- The bead tubes containing the filters can be stored at -80°C until processed.

DNA Extraction from Membrane Filters Using a Vacuum Manifold

Apparatus/Supplies

- **MoBio PowerSoil Kit** (Cat# 12888-100)
  - Each item in the kit is also sold separately
- **MoBio PowerVac Manifold Mini System** (MoBio # 11992), includes manifold adapters for the spin filer columns
- **MoBio vacuum manifold adapters** (Cat# 11992-20), needed if you use another manifold system such as Qiagen, always check to see if system parts are compatible
- **Vacuum pump** (needs to pull between 20 and 30 in Hg before opening any stopcocks)
- **5ml centrifuge tube** (Fisher Cat# 03391170)
- **DNA away** (VWR Cat# 53509-506)

- **10% Bleach**

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation through latex gloves and with inhalation of fumes.

Procedures

IMPORTANT NOTE: CHANGE GLOVES FOLLOWING ANY KNOWN OR SUSPECTED CONTACT WITH NON-STERILE LIQUIDS OR SOLIDS!! USE BARRIER PIPET TIPS THROUGHOUT!

Please see "Membrane Filtration for Environmental Samples SOP" for preparation of filters before DNA extraction.

I. Preparation for Extraction (with MoBio PowerSoil Kit)

- Spray bench with (1) 10% bleach and wipe, and (2) DNA Away and wipe after each use.
- Prepare one extraction blank (PowerBead tube with no sample).
- Each sample, including the extraction blank, should have a unique ID that is carried through the extraction process. Label each 2.0 ml tube top and filter column to maintain sample identity.
- Prepare a set of 2.0 ml tubes (as many tubes as samples, including the extraction blank) containing 250µl solution C2; put aside for step 4.
- Prepare another set of 2.0 ml tubes containing 285µl solution C3; set aside for step 7.
- Prepare 5 ml tubes containing 1.6 ml of C4; set aside for step 11. If there are two filters for one sample (due to turbidity and clogging during membrane filtration) these will be combined in step 11 by filtering both through the one spin filter column.

II. Extraction (Modified from Manufacturers instructions)

1. After placing filter aseptically into PowerBead tube, gently vortex, then add 60 µl of solution C1and vortex again, or invert several times.
2. Bead beat at 4000 rpm for 40 sec in a FastPrep Cell Disrupter (Thermo Savant).
3. Centrifuge tubes at 10,000 x *g* for 30 sec.
4. Transfer all supernatant to tube containing 250µl of solution C2 and vortex for 5 sec.
5. Incubate 4ºC for 5 min.
6. Centrifuge tubes at 10,000 x *g* for 1 min.
7. Transfer all supernatant to tube containing 285µl of solution C3, vortex briefly.
8. Incubate at 4ºC for 5 min.

   *WHILE WAITING: setup vacuum manifold with the vacuum adapters and a spin filter column for each sample. Snap the lid of a 2 ml tube, or place a

small piece of virgin foil, on top of each spin filter column. Do not forget to label each column with the correct sample ID.

9. Centrifuge tubes at 10,000 x *g* for 1 min.
10. Transfer all supernatant to a 5ml tube containing 1.6 ml solution C4 and vortex for 5 sec.
11. Turn on the vacuum and continuously load ≤ 600 µl in spin filter column until all remaining supernatant has passed through the spin filter (sample should not reach the rim at any time). Continue for each sample. Turn off vacuum stopcocks when sample has passed through and you are waiting for the next step.
    *IF SPIN FILTER COLUMN CLOGS, it can be placed into a 2 ml tube and microcentrifuged at 10,000 x *g* for 1 min. Place filter column back on vacuum to continue the extraction, or finish the extraction using microcentrifugation.
12. Load 750 µl of solution C5 to the spin filter column and open vacuum stopcock.
13. Once all solution has passed through the column: turn off the vacuum, detach spin columns, and place into 2.0 ml tubes.
14. Centrifuge at 14,000 rpm for 3 min to completely remove C5.
    WHILE WAITING: label clean 2.0ml for final spin; see step 17
15. Carefully place spin column into a new clean microcentrifuge tube.
16. Add 100 µl of solution C6 to the center of the white filter membrane.
17. Let sit at room temperature for 5 minutes.
18. Centrifuge at 10,000 x *g* for 30 sec.
19. Store eluate at -20°C.

Polymerase Chain Reaction (PCR) for human-associated Bacteroidales, *Methanobrevibacter smithii* and Human Polyomavirus

Apparatus/Supplies

- **2X GoTaq® Green Master Mix** (Fisher Cat# PRM7122 Or PAM7123)
- **10 µM Forward and reverse primer** (make 1:10 dilution of stock 100mM solution with nuclease free water; can order from www.idtdna.com)
- **Nuclease free/ PCR grade water-** comes with GoTaq mix or can be purchased separately (Fisher Sci cat # bp2484-50)
- **0.2 ml Thin walled PCR tubes** (USA Sci Cat# 1402-8100)
- **0.5 ml Tube** (USA Sci Cat# 1605-0099)
- **DNA away** (VWR cat# 53509-506)
- **Thermocycler**

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation with skin contact and inhalation of fumes.

Procedures

Preparation for handling DNA and PCR reagents

1. Decontaminate your work area with 70% alcohol followed by DNA away. Wipe away with a paper towel after each.
2. Turn the UV light on in the PCR workstation. It is programmed to remain on for 15 min. Tip boxes, pipettes, sharpie, vortex or centrifuge should be in the workstation if possible.

Primers

Primers are purchased with a 25nmole 'concentration' and standard desalting. They are delivered to the lab lyophilized and need to be rehydrated to a 100 µM concentration before use.

1. Add 10 times more water than the nmol concentration indicated on the tube. Eg. If the concentration of the primer in the tube is 32.70 nmol, resuspend with 327µl water. Use PCR grade water. This will make a *100µM stock solution*.
2. Store this solution in the -80°C freezer.
3. For your PCR reactions you will make a *10µM primer working solution* by diluting the stock solution 1:10 with water- usually 90µl water and 10µl stock solution. If you know you will be using this up quickly, you can make more than 100 µl working solution at a time, or several tubes of 100 µl, to avoid repeated freeze/thawing of the stock solution.

Master Mix

Typically, a master mix is made containing enough of each reaction component for all the reactions to be run with the primer set being used. A standard PCR reaction must contain Taq polymerase, Taq buffer, $MgCl_2$, Primer, deoxynucleotides (dATP, dCTP, dGTP and dTTP) and template DNA. The GoTaq green Master Mix being used will contain the Taq polymerase, buffer, $MgCl_2$ and dNTP's in a 2X concentrated solution so all you need are the primers, water to dilute the concentration of the GoTaq green in the final solution to 1X and template DNA.

1. Prepare the PCR workstation as described above under **Preparation for handling DNA and PCR reagents.**
2. Place the necessary number of 0.2ml PCR tubes and 1.5/2.0ml tubes (for making the master mix) into racks and place, open into the crosslinker. Include PCR tubes for a positive and negative control as well as your samples. Using the crosslinker (set to optimal crosslink; approx. 66µW/cm^2), crosslink the necessary tubes in racks twice. Before removing from the crosslinker, close all the tubes using new gloves.
3. Take your DNA and PCR reagents out of the freezer to thaw in separate crosslinked tube racks. Handle the PCR reagents before the DNA- you don't want to get DNA on your gloves and pass it onto your reagent tubes. Leave the DNA

rack on the bench where you will be adding the DNA to your PCR tubes and leave the PCR reagents in the PCR workstation.

4. Crosslink a cold block to hold your PCR reagents once they have thawed, twice.
5. Calculate the reagent volumes you will need for your assay PCR master mix- see PCR master Mix tables for each assay below. Include extra reactions for the PCR positive and negative controls as well as one extra reaction to account for pipette error.
6. In the PCR workstation, combine reagents in the 1.5/2.0 ml tubes mixing with the pipet after the addition of each new reagent.
7. Aliquot 23 µl into each PCR tube while in the workstation.
8. When ready to add DNA, move PCR tubes in rack out of the workstation to the DNA hood and add 2 µl template. Make sure to vortex tubes of DNA before taking from them. As you add the DNA, be sure to pipette up and down to mix the DNA with the master mix. Nothing is added to the PCR negative control tube, this tube will have a final volume of 23 µl.
9. Load tubes into the thermocycler and start the cycling program.
10. Once completed, reactions can be stored at -20°C until visualized by electrophoresis.
11. PCR products can be visualized following details below for each assay. The expected amplicon size is given below for each assay.

Human Polyomavirus Assay (HPyVs)

PCR Master Mix

Each 25 µl reaction contains:

Reagent	**volume**
GoTaq Green Master Mix (Promega)	12.5 µl
Forward primer (10µM)	1.0 µl
Reverse primer (10µM)	1.0 µl
Sterile PCR grade water	8.5µl
Template	2.0 µl

Thermocycler program:

Initial denaturing	94 C 2 min
Denaturing	94 C 20s
Anneal	55 C 20s
Extension	72 C 20s
Final extension	72 C 2 min
Number of cycles	45

Visualize amplicon by loading 10 µl into a 2% agarose gel stained with ethidium bromide. Run gel with 50 bp DNA molecular weight marker.

Primer references:

Askamit, A. J. 1993. PCR detection of JC virus. In T. F. S. D.H. Persing, F.C. Tenover, T.J. White (ed.), Diagnostic molecular microbiology principles and applications. Mayo Foundation, Rochester.

McQuaig, S. M. 2009. T. M. Scott, J. O. Lukasik, J. H. Paul & V. J. Harwood. Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. *Appl Environ Microbiol* **75**: 3379-3388.

Forward primer - SM2: 5'-AGT CTT TAG GGT CTT CTA CCT TT-3'
Reverse primer – P6: 5'-GGT GCC AAC CTA TGG AAC AG-3'
Amplicon size: 172bp

Human-associaed *Bacteroidales and Methanobrevibacter smithii* assays

Human-associaed *Bacteroidales* PCR Master Mix
Each 25 µl reaction contains:

Reagent	**volume**
GoTaq Green Master Mix (Promega)	12.5 µl
HF183 forward primer 10 µM	1.0 µl
Bac708 reverse primer 10 µM	1.0 µl
PCR grade sterile water	8.5 µl
Template	2.0 µl

***Methanobrevibacter smithii* PCR Master Mix**
Each 25 µl reaction contains:

Reagent	**volume**
GoTaq Green Master Mix (Promega)	12.5 µl
Mnif forward primer 10 µM	1.0 µl
Mnif reverse primer 10 µM	1.0 µl
PCR grade sterile water	8.5 µl
Template	2.0 µl

Diluting template (1:10, 1:100 with PCR grade sterile water) can help if inhibitors are present.

Thermocycler program (touchdown) for both human *Bacteroidales* and *M. smithii*:

- Initial denaturing 94°C 3 min 30 sec
- 94°C for 45 sec; 45 sec at 65–55°C (step down 1°/2 cycles from 65 to 62°C & 1°/cycle from 62 to 55°C); 72°C 30 sec
- 30 cycles: 94°C 45 sec, 55°C 45 sec, 72°C 30 sec
- Final extension: 72°C 5 min

The GoTaq Green Master Mix has dye already added, so we directly load 8 µl of the PCR amplicons to a 1% agarose gel and stain with ethidium bromide. Please use 100bp ladder.

Primer references:

Bernhard, A. E., and K. G. Field. 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. Appl Environ Microbiol 66:4571-4574.

183f: 5'ATC ATG AGT TCA CAT GTC CG 3'
708r: 5'CAA TCG GAG TTC TTC GTG 3'
Amplicon size: 525 bp

Ufnar, J. A., S. Y. Wang, J. M. Christiansen, H. Yampara-Iquise, C. A. Carson, and R. D. Ellender. 2006. Detection of the nifH gene of Methanobrevibacter smithii: a potential tool to identify sewage pollution in recreational waters. J Appl Microbiol 101:44-52.

Mnif-342f: 5'AAC AGA AAA CCC AGT GAA GAG 3'
Mnif-363r: 5'ACG TAA AGG CAC TGA AAA ACC 3'
Amplicon size: 222 bp

Revision History

Revision	Revision Date	Approval Date	Prepared By	Description of Revision
1	12/06/10		K. Gordon	Initial compilation